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Short Communication

6-Pyruvoyl Tetrahydropterin Synthase in Human Tissues and Cell Lines

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Introduction

6-Pyruvoyl tetrahydropterin synthase (PTP synthase) is one of the key enzymes in the biosynthetic pathway of tetrahydrobiopterin (BH_4) (1), the natural cofactor of the mammalian aromatic amino acid hydroxylases (2). PTP synthase deficiency is the most common and heterogeneous genetic disease in BH_4 metabolism. Analysis of urinary pterins combined with the measurement of enzyme activity in red blood cells, as well as analysis of metabolites in CSF enables differentiation between the variants of PTP synthase deficiency.

In this paper we describe the use of specific monoclonal antibodies prepared against human piutitary gland PTP synthase for the immunocytochemical localization of the enzyme in bone marrow smears, different cell lines, human cultured skin and amniotic fluid fibroblasts, as well as in human and mouse macrophages. We also report the quantification of PTP synthase activity in extracts of the above mentioned cells.

Utilizing specific anti-PTP synthase monoclonal antibodies and an enzyme assay based on the quantification of biopterin using fluorescence detection, amniotic fluid fibroblasts may be suitable for prenatal diagnosis of PTP synthase deficiency.

Material and Methods

Pteridines and other chemicals as well as tissue culture mediums were obtained as previously described (3).

In vitro *immunization and subcloning by limited dilution*

Monoclonal antibodies were produced using the *in vitro* immunization technique with the antigen blotted from SDS-PAGE to Immobilon polyvinylidene difluoride (PVDF) membranes (Guzman *et al.* submitted). PTP synthase partially purified from human pituitary gland was used as antigen. Fusion, cell culture, HAT selection and cell cloning by limited dilution were done as reported previously (3).

The enzyme linked immunosorbent assay (ELISA) test for specific antibodies was performed according to standard procedures with slight modifications.

PTP synthase assay

The PTP synthase activity assay was performed as described previously (4), except for the incubation time, which was reduced to 20 minutes. One unit of enzyme activity catalyses the production of 1 µmol BH_4/min from dihydroneopterin triphosphate (NH_2TP) at 37 °C under standard conditions.

Tissue culture

The human transitional cell bladder carcinoma line T 24 (obtained from Dr. E. Werner, Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Austria), human skin fibroblasts and the human neuroblastoma cell line SK-N-BE were cultured in minimum essential medium supplemented with

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Abbreviations: BH_4, tetrahydrobiopterin; PTP synthase, 6-pyruvoyl tetrahydropterin synthase; ELISA, enzyme linked immunosorbent assay; PVDF, polyvinylidene difluoride; HAT, hypoxanthine, aminopterin, thymidine; NH_2TP, dihydroneopterin triphosphate; APAAP, alkaline phosphatase monoclonal anti-alkaline phosphatase complex; LPS, lipopolysaccharide.

10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 ng/ml steptomycin. The pituitary cell line GH4C1 (obtained from Dr. W. Schlegel, Fondation pour Recherches Médical, Université de Genève, Switzerland), a subclone from the cell line GH3, was cultured in Nutrien mixture F-10 (HAM) supplemented with 2.5% fetal calf serum and 15% (Donor) horse serum. The human Burkitt lymphoma cell lines BJA-B and Namalwa (obtained from Dr. E. Schreiber, Department of Clinical Immunology, University Hospital, Zürich, Switzerland) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 ng/ml streptomycin and 50 μM 2-mercaptoethanol.

Human and mouse macrophages were generously supplied by Dr. A. Schaffner (Department of Internal Medicine, University Hospital, Zürich, Switzerland). The PTP synthase activity was measured in the cytosol fractions of mouse peritoneal macrophages and monocytes purified from human peripheral blood (99% purified).

Preparation of the cell extracts

Adherent cells were detached by trypsin (0.5 mg/ml) in phosphate-buffered saline (PBS, pH 7.4) and washed 3 times with Hanks' balanced salt solution. Cells were then suspended in 600 – 1000 μl of 5 mM Tris/HCl, pH 7.4, and disrupted by freezing and thawing. After centrifugation at 12000 *g* for 15 minutes, 50 μl of the supernatant were used for the PTP synthase activity assay. The non-adherent cell lines were washed 3 times with Hanks' balanced salt solution and then disrupted as described above.

Preparation of the cell smears

Bone marrow smears were routinely prepared from healthy controls. Adherent cells such as skin fibroblasts and other above mentioned cell lines were cultured in Nunc Slide Flasks for 4 days. Non-adherent cells were cytocentrifuged. The slides were washed twice with Hanks' balanced salt solution and then air dried overnight. All smears were stored at −20 °C, protected from humidity, or directly used for the immunocytochemical localization of the enzyme.

Cytocentrifuged smears of the THP-1 myelomonocytoma cell line were obtained from Dr. G. Werner-Feldmayer (Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Austria).

Immunoenzymatic staining of PTP synthase

The slides were fixed for 1 minute in acetone/methanol 1 : 1 and then processed as described (3), using anti-PTP synthase monoclonal antibodies and the alkaline phosphatase/monoclonal anti-alkaline phosphatase labeling technique (APAAP) (5). Positive and negative controls for the immunostaining of the cell smears were the same as reported previously (3). As positive control for the human cultured macrophages and for the THP-1 cell line, a monoclonal antibody to a surface antigen from monocytes was used. The negative control was the same as for the above mentioned cell smears.

Results and Discussion

In contrast to previous results (6) we were now able to measure PTP synthase activity in human macrophages (0.2 μU/mg protein). These results are higher than those reported by Werner *et al.* (7) (0.1 μU/mg protein). However, even higher PTP synthase activity (0.4 μU/mg protein) was obtained by Dr. Shintaku in our department (unpublished data). No enhancement of activity was found when human macrophages were stimulated with lipopolysaccharide (LPS) (data not shown). With the mouse macrophages, a 2-fold higher PTP synthase activity was found. Surprisingly, very intense staining was found in human macrophages with the monoclonal antibody 16G6 as compared with cell lines that showed higher PTP synthase activity. Similar strong staining of PTP synthase with monoclonal antibody 16G6 was found in the bone marrow smears of healthy donors.

As previously reported (3), the cytosol of skin fibroblasts is uniformly stained for PTP synthase and very intense staining of the cytoplasm of dividing fibroblasts was observed. We further investigated PTP synthase activity in fibroblasts after disruption by freezing and thawing. A PTP synthase activity of 4 μU/mg protein was found. The lack of PTP synthase activity described earlier (8) was due to incomplete lysis of the cells, rather than to sensitivity problems. We also observed an enhancement of activity when the cells were incubated for 15 min with 1% Triton-X-100 in 5 mM Tris/HCl, pH 7.4, at 4 °C. It is not yet clear if this enhancement is due to better disruption of the cells or if the PTP synthase is also membrane bound.

As shown in Table 1 high STP synthase activity was found in the neuroblastoma cell line SK-N-BE (24.3/23.8 μU/mg protein), in the Burkitt lymphoma cell line BJA-B (32/31.4 μU/mg protein), in the human T

Table 1. PTP synthase activity in different cells.

Cells	Activity μU/mg protein	Ref.	n
SK-N-BE	24.3/23.8		2
BJA-B	32.0/31.4		2
GH4C1	38.6		1
Namalwa	n.d.		5
Molt-4	48.0		1
THP-1	0.397 ± 0.036	7	5
	0.349 ± 0.055*	7	5
T-24	7.63 ± 0.77	7	5
	7.86 ± 0.34*	7	5
Fibroblasts	4.0 ± 0.12		7
Macrophages			
human	0.2**		1
mouse	0.4**		1

One unit of enzyme activity catalyses the production of 1 μmol BH_4/min from NH_2TP at 37 °C under standard conditions.
* Treatment of the cells with IFN gamma
** No difference was observed after treatment with LPS
n.d. = not detected

cell leukemia line Molt 4 (48 μU/mg protein) as well as in the pituitary gland cell line GH4C1 (38.6 μU/mg protein). Surprisingly we did not find PTP synthase activity in the Namalwa cell line (another Burkitt lymphoma cell line).

The cross reactivity of the 16G6 and 7F8 antibodies with PTP synthase in those cell lines was also investigated. As expected, the cells presented uniform staining for PTP synthase and no unspecific staining with the negative control was found.

With cytocentrifuged smears of the cell lines T 24, Molt 4, and THP-1, uniform staining for PTP synthase with monoclonal antibody 16G6 was also observed. PTP synthase activity in the T 24 and THP-1 cell lines has already been reported by Werner *et al.* in 1990 (7).

Prenatal diagnosis of PTP synthase deficiency has been routinely performed by the measurement of pteridines in amniotic fluid and PTP synthase activity in fetal blood.

As reported previously for blood smears (5), very heterogeneous weak labeling for PTP synthase was found in erythrocytes while reticulocytes were clearly and intensively stained. We also demonstrated that T-lymphocytes and granulocytes presented intense and clear staining for PTP synthase. Because of the variable cell counts for white cells, reticulocytes and young erythrocytes, the range of PTP synthas activity has relatively great variability. Furthermore, fetoscopy can be performed only in specialized hospitals and may be a risk for the fetus.

In one effort to develop a suitable assay for prenatal diagnosis of PTP synthase deficiency, we measured PTP synthase activity in the amniotic fluid cells of four healthy donors (kindly supplied by Dr. Binkert, Department of Medical Genetics, University of Zürich, Switzerland). We were able to detect PTP synthase activity in cultured amniotic cells (data not shown). Therefore, measurement of PTP synthase activity in cultured amniotic fluid fibroblasts may offer a possibility for prenatal diagnosis of PTP synthase deficiency in pregnancies at risk. Since immunocytochemistry is much more sensitive than fluorometric detection of biopterin, we plan to visualize PTP synthase in uncultured and cultured amniotic cells of healthy donors.

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